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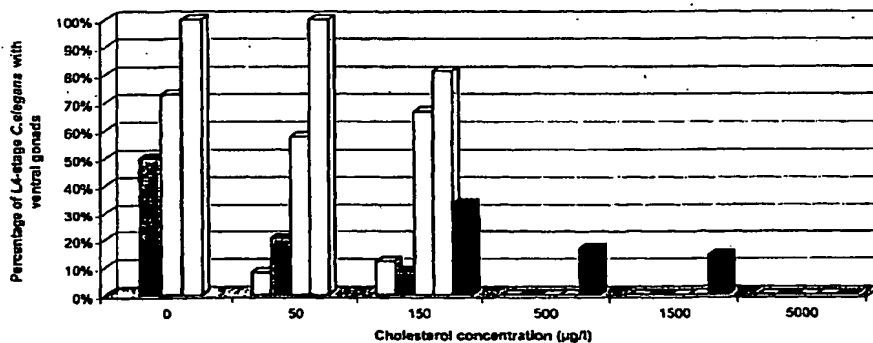
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- (54) Title: METHOD FOR DETERMINING THE INFLUENCE OF A COMPOUND ON CHOLESTEROL TRANSPORT

Effect of Dehydroxy-O-methyl-Ezetimibe and Cholesterol concentration on the presence of ventral gonads in *C. elegans*.

□ Blanco ■ DMSO □ 5 µM Dehydroxy-O-methyl-Ezetimibe
 □ 50 µM Dehydroxy-O-methyl-Ezetimibe ■ 500 µM Dehydroxy-O-methyl-Ezetimibe

- (57) Abstract: The invention relates to methods for determining the influence of a compound on cholesterol handling, and in particular on cholesterol transport across biological membranes. In particular, the invention relates to a method for determining the influence of a compound on the uptake of cholesterol by a nematode worm, which method is representative for cholesterol transport across (other) biological membranes.

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Method for determining the influence of a compound on cholesterol transport.

The present invention relates to a method for determining the influence of a compound on cholesterol handling, and in particular on cholesterol transport across biological membranes.

Specifically, the present invention relates to a method for determining the influence of a compound on the uptake of cholesterol by a nematode worm, which method is representative for cholesterol transport across (other) biological membranes.

Further aspects, embodiments, advantages, and/or uses of the invention will become clear from the further description below.

It is well known that cholesterol handling, and in particular the uptake of cholesterol by certain cells and/or tissues such as the cells that form the walls of blood vessels, plays an important part in many diseases, such as cardiovascular disease (for example arteriosclerosis) and obesity. Because of this, it is expected that compounds that act to alter and/or influence the transport of cholesterol across biological membranes - for example compounds that can reduce cholesterol uptake by cells or tissues - will be useful therapeutic agents for use in these disease areas. One example of such a compound influencing cholesterol handling is ezetimibe, which is used in the treatment of cardiovascular diseases.

Thus, there is a need for methods that can be used to identify and/or develop compounds that influence cholesterol handling. More in particular, there is a need for methods that can be used to determine the influence of a compound on cholesterol transport across a biological membrane.

The present invention provides such a method, in which the influence of a compound on cholesterol transport is determined using a nematode worm, in particular a nematode worm of the species *C. elegans*.

It is well known that nematodes such as *C. elegans* need to take up cholesterol from their surroundings, and that lack of sufficient cholesterol in the medium can lead to biological defects, such as abnormal development of the gonads (e.g. the formation of ventral gonads), retarded growth and development, and defects in reproduction (e.g. sterility and reduced brood size); reference is made to, for example, Matyash et al., Molecular Biology of the Cell, Vol.12, 1725-1736, June 2001.

Thus, in principle, compounds that inhibit cholesterol uptake by the nematode worm should cause such defects, even in the presence of sufficient cholesterol in the medium. However, in their research leading up to the present invention, the inventors have found that at concentrations of cholesterol in the medium of more than 500 µg/l, the presence of 1-(4-fluorophenyl)-3-[3-(4-fluorophenyl)propyl]-4-(4-methoxyphenyl)-azetidin-2-one (also referred to herein as "*dehydroxy-O-methyl-ezetimibe*"), an analog of ezetimibe known to inhibit cholesterol transport (vide S.B. Rosenblum et al., J. Med. Chem. 1998, 41, 973-980), in concentrations up to 500 µM has no significant influence on nematode worms.

The present invention is based on the finding that the influence of a compound on cholesterol transport across a biological membrane can be determined by determining the uptake of cholesterol by a nematode worm from a medium, with and without the presence of said compound, provided that the concentration of cholesterol in the medium is less than 500 µg/l. As can be seen from the Example below, it is not possible to measure the influence of the compound when the concentration of cholesterol in the medium is above 500 µg/l.

Thus, in a first aspect, the invention relates to a method for determining the influence of a compound on the uptake of cholesterol by a nematode worm from the medium, comprising the steps of:

- a) incubating the nematode worm in a suitable medium, wherein said medium contains: (i) the compound; and (ii) cholesterol in a concentration of less than 500 µg/l;
- b) determining at least one cholesterol-relevant phenotype of the nematode worm.

Preferably, in step a), the concentration of cholesterol in the medium is less than 400 µg/l, preferably less than 350 µg/l, more preferably less than 300 µg/l, for example about 250 µg/l, 200 µg/l, 150 µg/l, 100 µg/l, 50 µg/l, 10 µg/l or (essentially) zero. Such a medium will also be referred to below as a "*low-cholesterol medium*".

It should be noted that instead of cholesterol, also a suitable cholesterol derivative or analog may be used, such as 24-hydroxy-cholesterol or 24,25-dihydroxy-cholesterol or similar compounds, as well as suitably labeled derivatives of cholesterol, such as fluorescently labeled cholesterol (for example 25-NDB cholesterol). Thus, the term "cholesterol" as used herein in its broadest sense also includes the use of one or more of such derivatives and/or analogs, as long as the total

concentration of cholesterol and cholesterol derivatives/analogs in the medium is within the ranges indicated above. Preferably, however, only cholesterol is used.

The nematode worm used is preferably of the genus *Caenorhabditis*, in particular *Caenorhabditis elegans* ("*C. elegans*"). For general information on *C.*

5 *elegans* and techniques for handling this nematode worm, reference is made to the standard *C. elegans* handbooks, such as W.B. Wood et al., "*The nematode Caenorhabditis elegans*", Cold Spring Harbor Laboratory Press (1988); D.L. Riddle et al., "*C. ELEGANS II*", Cold Spring Harbor Laboratory Press (1997); "*Caenorhabditis elegans, Modern Biological analysis of an organism*": ed. by H. Epstein and D. Shakes, Methods in Cell Biology, Vol 48, 1995; and "*C. elegans, a practical approach*", ed. by I.A. Hope, Oxford University Press Inc. New York, USA, 1999, as well as to the following applications by Applicant: PCT/EP99/09710 (published on 15 June 2000 as WO 00/34438); PCT/EP99/04718 (published on January 15, 2000 as WO/00/01846); PCT/IB00/00575 (published on October 26, 2000 as WO 00/63427); PCT/IB00/00557 (published on October 26, 2000 as WO 00/63425); PCT/IB00/00558 (published on October 26, 2000 as WO 00/63426); as well as in for instance PCT/US98/10080 (published on 19-11-1998 as WO 98/51351), PCT/US99/13650, PCT/US99/01361 (published on 29-07-1999 as WO99/37770), and PCT/EP00/05102.

20 In the invention, any suitable mutant, line or strain of *C. elegans* can be used, including but not limited to wildtype, N2, and suitable mutants or transgenic strains or lines, such as the mutant strains or lines mentioned in the *C. elegans* handbooks and in the applications by Applicant referred to above. Usually, the use of wildtype or N2 is preferred. However, the invention also includes the use of strains or
25 mutants in which, compared to wildtype or N2, at least one cholesterol-relevant phenotype (as defined herein) is altered; and/or the use of strains or mutants which, again compared to wildtype or N2, have altered properties with respect to the handling, uptake, use and/or transport of cholesterol, such as mutants which are (hyper)sensitive to cholesterol deprivation, again compared to wild-type or N2. An
30 example of the latter is the *npc-1* mutant described by Sym et al., Current Biology, Vol.10, no.9, 527-530.

In another embodiment, the strain used is a beta-glucuronidase mutant, and in particular a mutant with reduced glucuronidase activity (again compared to wildtype

or N2), such as the *gus*-mutants, for example *gus-1* (Sebastiano et al., Genetics 112: 459-468 (1986)).

In another embodiment, the strain is a strain with a phenotype of constitutive pharynx pumping, such as the strain HD8 described in the International applications
5 WO 00/63427 and WO 00/63425, both by applicant.

In yet another embodiment, the mutant is a *daf*-mutant, such as *daf-2* mutant, for example the *daf-2* mutants mentioned in the International application WO 01/93669 by applicant, and in particular the *daf-2* mutant *daf-2(m41)*.

Usually, a sample of nematodes is used, such as between 2 and 500 or more, in
10 preferably between 3 and 300, more preferably between 5 and 200, even more preferably between 10 and 100 nematodes per sample. However, it is also within the scope of the invention to use (a sample consisting of) a single worm. It should also be noted that during step a) of the method of the invention, the size of the sample may change, e.g. due to reproduction and/or death.

15 The medium used can be any solid, semi-solid and/or liquid medium suitable for maintaining and/or cultivating nematode worms, such as agar plates, NGM plates, M9, S-buffer or a viscous medium with viscosity greater than M9 (e.g. as described in PCT/IB00/00575 and other applications by Applicant, and as measured at the cultivation and/or incubation temperature used, e.g. using an Ostwald, Ubbelohde or
20 Brookfield viscosimeter), on a plate, in a petri-dish, in a tube, in a flask, in the well of a multi-well plate, or in another suitable vessel or container. Reference is again made to the *C. elegans* handbooks and applications by Applicant referred to above.

The medium can be prepared in a manner known per se, by incorporating the compound(s) in the concentration to be tested, and carefully controlling the amount of
25 cholesterol in the medium to within the ranges mentioned above. It is within the scope of the invention that the medium contains some cholesterol, e.g. added cholesterol and/or cholesterol that is derived from the bacteria used as a food source for the nematodes, provided the total amount of cholesterol in the medium is controlled to within the ranges mentioned above.

30 The nematodes are cultured at a suitable temperature, which will generally be in the range of 15-30°C, in particular in the range of 17-27°C, and usually about 20 to 25 °C; and the presence of a suitable source of food such as bacteria - for example a suitable strain of *E.coli* such as *E. coli* OP 50 CS2 - in an amount of between 0.05 and 0.5 % w/v, preferably about 0.125 % w/v.

The invention is not specifically limited as to the compound(s) that may be tested.

For example, although the compound is preferably soluble in the medium used (at least at the concentrations used for the assay), the compound may also be present
5 in the medium as discrete liquid, semi-solid or solid particles that are ingested by the worms (for example when the worms feed); or the particles may be present in a suitably dispersed or emulsified form. The compounds may also be added to the medium as a solution in a suitable solvent, such as dimethylsulfoxide (DMSO).

Preferably, the compounds should be such that, at the concentrations and
10 during the period of time and further conditions used in the assay, they allow at least some of the worms to develop so as to reach the stage in their life cycle where the cholesterol-relevant phenotype can be determined; and/or allow the worms to reproduce and their offspring to reach the stage in their life cycle where the cholesterol-relevant phenotype can be determined. It should be noted, however, that
15 the compounds tested may, compared to wild type or the specific strain used, affect (and in particular delay) development and/or affect (and in particular reduce, but not completely eliminate) fertility, brood size and/or egg hatching, and that this is within the scope of the invention.

In one preferred embodiment, the compound(s) to be tested are "small
20 molecules", by which is generally meant herein a molecular entity with a molecular weight of less than 1500, preferably less than 1000. This may for example be an organic, inorganic or organometallic molecule, which may also be in the form of a suitable salt, such as a water-soluble salt; and may also be a complex, chelate and/or a similar molecular entities, as long as its (overall) molecular weight is within the range
25 indicated above.

In a preferred embodiment, such a "small molecule" has been designed according to, and/or meets the criteria of, at least one, preferably at least any two, more preferably at least any three, and up to all of the so-called Lipinski rules for drug likeness prediction (vide Lipinski et al., Advanced Drug Delivery Reviews 23 (1997),
30 pages 3-25). As is known in the art, small molecules which meet these criteria are particularly suited (as starting points) for the design and/or development of pharmaceuticals for human use, and may for instance be used as starting points for hits-to-leads chemistry, and/or as starting points for lead development (in which the methods of the invention may also be applied).

Also, for these purposes, the design of such small molecules (as well as the design of libraries consisting of such small molecules) will preferably also take into account the presence of pharmacophore points, for example according to the methods described by I. Muegge et al., J. Med. Chem. 44, 12 (2001), pages 1-6 and the

5 documents cited herein.

In another embodiment, the compound(s) may be "small peptides", which term is meant to generally cover (oligo)peptides that contain a total of between 2 and 35, such as for example between 3 and 25, amino acids (e.g. in one or more connected chains, and preferably a single chain). It will be clear that some of these small

10 peptides will also be included in the term small molecule as used herein, depending on their molecular weight.

In one preferred, but non-limiting embodiment, the invention is used to screen a set or library of (related or otherwise unrelated) small molecules, for example a standard "robustness set", a primary screening library (e.g. of otherwise unrelated

15 compounds), a combinatorial library or a series of closely related chemical analogs, etc.. Such sets or libraries will be clear to the skilled person, and may for instance include, but are not limited to, such commercially available chemical libraries such as the various libraries available from Tocris Cookson, Bristol, UK. Such libraries may be screened manually in the assay of the invention, or automated screening techniques

20 may be used, such as those mentioned in the prior art on the use of *C. elegans* cited above.

For example, in one non-limiting embodiment, the compounds may be (chosen from) a series of chemical analogs of ezetimibe, although the invention in its broadest sense is not limited to the specific compound(s) tested.

25 The concentration of the compounds to be tested in the medium is not critical, and can generally be between 0.01 and 10.000 μM or more, in particular between 1 and 1000 μM , such as about 5, 10, 50, 100, 200, 300, 400 or 500 μM . In this embodiment, it is also possible to test a compound at two or more different concentrations, e.g. to establish a dose-response curve, and/or to test two or more

30 compounds on a single sample of worms, e.g. to determine whether these compounds have a synergistic effect. Also, a medium without any compound(s) to be tested, and/or a medium containing a predetermined concentration of a known enhancer and/or known inhibitor of cholesterol transport (such as dehydroxy-O-methyl-ezetimibe used in the Examples below) may be used as a reference.

In another embodiment, the compound is a nucleic acid, for example a DNA, an RNA or a PNA. The nucleic acid may be single stranded or double stranded, and may also be in the form of a construct, such as a vector or plasmid.

In one non-limiting embodiment, the nucleic acid may encode a nucleotide sequence that, when taken up by the worm, alters (i.e. increases or decreases) the level of expression of one or more genes in said worm, compared to wild type/N2 and/or to the specific strain used. In particular, the compound may be a nucleic acid encoding a nucleotide sequence that, when taken up by the worm, partially or totally reduces (i.e. "downregulates", "knocks down" or "knocks out") the expression of one or more genes, compared to wild type or to the specific strain used. As such, it may for example be an antisense molecule and in particular a double stranded RNA molecule, more in particular a dsRNA molecule that encodes (at least part of) the sequence of the gene(s) to be downregulated, i.e. by means of so-called RNA interference or "RNAi", for which reference is made to for example WO 99/32619, as well as to the applications WO 00/01846 and WO01/88121 by applicant, incorporated herein by reference. Generally, such RNAi techniques involve contacting the nematode worm(s) with a one or more double stranded RNA (dsRNA) fragments corresponding to the gene(s) of interest. This can be performed in any suitable manner known per se, such as by injection, soaking or feeding with the dsRNA, as will be clear to the skilled person. In a particularly preferred embodiment, the nematode worms are contacted with the dsRNA by feeding the nematode worms with a bacterial strain that contains and/or that expresses (or are capable of expressing upon ingestion by the worms) the dsRNA fragment corresponding to the gene of interest. Such RNAi feeding techniques, as well as vectors, bacterial strains and/or bacterial libraries are *inter alia* described in the International applications WO 00/01846 and WO 01/88121 by Applicant, incorporated herein by reference. Also, libraries of *E.coli* feeding strains based on RNAi fragments for *C. elegans* genes that are suitable for use in the invention are commercially available, for example from MRC Geneservice (UK).

To determine the influence of the compound on the cholesterol uptake by the nematode worms, the sample of worms is incubated with the compound(s) to be tested in said low-cholesterol medium for a period of at least 2 hours, preferably at least 6 hours, more preferably at least 12 hours, even more preferably at least 24 hours. In particular, the nematode worms may be incubated for a period of at least 2 days,

preferably at least 3 days (e.g. to obtain F1 progeny), and up to 7 days or more, e.g. 10 days or more (e.g. to obtain F2 progeny).

Preferably, in the invention, the nematode worms are incubated with the compound(s) to be tested in the low-cholesterol medium for a period of time which spans at least two different development stages of the nematode, e.g. at least L3, L4 or adult stage.

For example, in the invention, the nematode worms may be incubated with the compound(s) to be tested in the low-cholesterol medium for a period of time which spans at least two different generations of the nematode, i.e. such that the original sample of nematodes (i.e. used as parent) are allowed to produce at least F1 progeny, and optionally also F2 progeny. For example, starting with L3 (preferred), L4 or adults of the parent generation, the nematodes may be incubated until the F1 progeny reaches L4 or adult stage, and/or until the F2 progeny reaches L4 or adult stage.

After incubation of the nematodes with the compound(s) to be tested in the low-cholesterol medium, in step b), at least one cholesterol-relevant phenotype of the nematode worms is determined.

By "cholesterol-relevant phenotype" is meant any detectable property of the nematode that is affected by, influenced by and/or otherwise representative for the amount of cholesterol taken up by the nematode used. Preferably, said detectable property is a detectable biological change, and in particular a detectable phenotypical, physiological, behavioral and/or biochemical change.

In particular, said "cholesterol-relevant phenotype" may be chosen from phenotypes that are dependent on or associated with cholesterol (e.g. cholesterol uptake and/or cholesterol handling) in the worm, such as phenotypes associated with development, reproduction; as well as biochemical and other changes as for example described by Matyash et al, *supra*. More in particular, said cholesterol-relevant phenotype may be chosen from growth (delay), brood size, the amount of ventral gonads formed, of which the latter is particularly preferred.

In *C. elegans*, the gonads are formed by migration of the distal tip cells (DTC's) during development, which migration consists of three linear phases punctuated by two orthogonal turns, resulting in mature gonads that extend in a U-shape from the ventral side via gonadal arms to the dorsal side. Reference is for example made to *C. elegans II (supra)*, pages 242-24603-604; Nishiwaki, Genetics 152: 985-987 (July 1999); Nishiwaki et al., Science, 288, 23 June 2000, 2205; Merz et

al., Genetics, 158: 1071-1080 (July 2001); Su et al., Development 127, 5885-594 (2000) and the references cited therein.

As used herein in its broadest sense, the term "*ventral gonad*" or "*ventral gonad phenotype*" is collectively used to indicate any phenotype in which there is a defect in said migration of the DTC's and/or (more generally) in the development of the germline, such that abnormal/misshapen gonads are formed, and in particular gonads that only extend on the ventral side on the nematode and/or that do not (fully) extend to the dorsal side of the nematode and/or that have abnormally thin or otherwise misshapen gonadal arms, and more in particular the former of the three.

Such ventral gonad phenotypes are well known in the art and have for example been described for the *C. elegans* mutants *unc-5*, *unc-6*, *unc-40*, *daf-12* and *mig-8* and similar mutants; reference is again made to the prior art cited in the previous paragraph (see for example Su et al., Figure 2 on page 588; and Nishiwaki, Genetics 152: 985-987 (July 1999)).

Usually, in the invention, said "*ventral gonad phenotype*" will be the result of a defect in the second, ventral-to-dorsal second phase of DTC migration, although the invention in its broadest sense is not limited to any specific biological or developmental mechanism that leads to the formation of ventral gonads, and other defects in the formation of the gonads (in each of the three phases) leading to the formation of ventral gonads have been observed in practice, for example depending on the specific conditions of the assay.

The term "*ventral gonad*" phenotype as used herein may also for example include the "*endomitotic oocyte*" or "*Emo phenotype*" described by Iwasaki et al., J. Cell Biol. 134: 699-714 and Shim et al., Molecular Reproduction and Development 61: 358-366 (March 2002).

The ventral gonad phenotype can easily be determined visually, e.g. using a light microscope or - if it is to be examined in more detail - using Nomarski microscopy.

The amount of ventral gonads formed in a sample (i.e. the number of worms in the sample that show a ventral gonad phenotype) may for example be expressed as a percentage calculated as [*Number of worms showing a ventral gonad phenotype*] divided by [*Total number of worms (i.e. worms with ventral gonads and the number of worms without ventral gonad)*] times [100%], in which the number of worms with ventral gonads and the number of worms without ventral gonads in a sample of

worms may again for example be determined visually, e.g. by counting. Visualization of the ventral gonad phenotype may also be facilitated by using a suitable staining protocol, for example a staining protocol that specifically stains cells in the gonads or any other suitable staining protocol; however, in practice this is usually not required.

5 In another preferred embodiment, the uptake of cholesterol by the nematode worms may be measured directly, for example by measuring the cholesterol levels in the nematode worm. For example, this may be performed using suitably labeled cholesterol and/or a suitably labeled cholesterol derivative (such as radioactively labeled cholesterol or fluorescently labeled cholesterol).

10 The cholesterol-relevant phenotype may be determined in any suitable manner known per se. For example, growth delay, brood size and formation of ventral gonads may be determined visually (usually under a microscope), whereas the uptake of labeled cholesterol may be determined using a scintillation counter.

15 The influence of the compound(s) to be tested is preferably such that, when the compound influences the uptake and/or handling of cholesterol by the nematode worm under the conditions used for the assay, this leads to an observable change in said cholesterol-relevant phenotype. By "observable change" is meant that the change in said cholesterol-relevant phenotype can be observed, detected and/or (qualitatively and/or quantitatively) measured/determined in a suitable manner
20 known per se, depending on the specific cholesterol-relevant phenotype.

 Also, to measure/determine said observable change in said cholesterol-relevant phenotype, any change in the cholesterol-dependent phenotype may be compared to the corresponding change (if any) in one or more suitable references/controls. As the control(s), any suitable worm or sample of worms can be
25 used, including but not limited to: a wild-type and/or N2 worm; a sample of the worms used for the assay but without the presence of the compound to be tested and/or that is exposed to a different concentration of the compound to be tested; a sample of worms that is exposed to a known concentration of a compound that is known to influence the uptake, handling, transport and/or regulation of the levels of
30 cholesterol in the worm (such as ezetimibe, dehydroxy-O-methyl-ezetimibe and/or one of the statin-type drugs); a mutant strain with one or more mutations in one or more genes that are known to be involved in the uptake, handling, transport and/or regulation of the levels of cholesterol; and/or a worm strain in which at least one gene

that is known to influence the uptake, handling, transport and/or regulation of the levels of cholesterol has been upregulated and/or downregulated.

For example, starting with a sample of L3 (preferred), L4 or adult parent worms, an increase in the percentage of ventral gonads, as determined in L4 (preferred) or in adults of the F1- and/or F2-progeny, and compared to a reference sample incubated without compound, is an indication that the compound to be tested acts as an inhibitor of cholesterol transport. Also, a decrease in the amount of labeled cholesterol taken up by the nematodes over a given period of time, compared to a reference sample incubated without compound, is an indication that the compound to be tested acts as an inhibitor of cholesterol transport.

As mentioned above, by using the uptake of cholesterol by nematode worms as a model, the method of the invention makes it possible to determine the influence of a compound on the transport of cholesterol across biological membranes, and/or that more generally influence the uptake, handling, transport and/or regulation of the levels of cholesterol. As such, it is expected that the method of the invention may for example be used in the pharmaceutical field, for instance to identify, test and/or develop compounds that influence cholesterol transport across biological membranes, for example across cell membranes in to cells or tissues. The method of the invention may also be used to investigate and/or identify biological elements - such as enzymes, channels and transporters - that are involved in (active) transport of cholesterol across biological membranes, for example for mode-of-action studies and/or for the identification of genes and/or pathways that are involved the uptake, handling, transport and/or regulation of the levels of cholesterol (for example by means of genetic screening techniques, e.g. involving EMS mutagenesis followed by outcrossing/selecting suitable mutants and cloning/isolating the gene(s) carrying the relevant mutation. In one embodiment, said screen may be configured to identify mutations that lead to an altered sensitivity to a compound that influences the uptake, handling, transport and/or regulation of the levels of cholesterol - such as ezetimibe or dehydroxy-O-ezetimibe - in order to identify biological elements with which said compound interacts). The elements thus identified may then for example be used as molecular targets for the development of pharmaceutically active compounds *in vitro* and/or for therapeutic intervention using such pharmaceutically active compounds *in vivo*.

Although the invention is not limited to any specific hypothesis or explanation, it is assumed that the use of concentrations of cholesterol in the medium below 500 µg/l make it possible to determine the influence of the compound(s) on the "active" (i.e. "compound-dependant") transport of cholesterol; whereas at concentrations of cholesterol in the medium above 500 µg/l, the influence of "passive" transport of cholesterol (for example passive diffusion through the cuticle, which is expected to be independent of the compound(s) to be tested) is such that this is no longer possible. Thus, the invention also provides an indication that the uptake of cholesterol by nematodes such as *C. elegans* is governed by an active mechanism, at least at low cholesterol concentrations.

The invention will now be illustrated by means of the following non-limiting Example and the Figure, which is a graph showing the influence of dehydroxy-O-methyl-ezetemibe on the percentage of ventral gonads in *C. elegans* N2 at different cholesterol concentrations.

Example:

4 female *C. elegans* N2 worms (young L4's) were incubated on NGM plates, at concentrations of dehydroxy-O-methyl-ezetemibe of 0 (blank reference), 5 µM, 50 µM and 500 µM, and at cholesterol concentrations of 0, 50, 150, 500, 1500 and 5000 µg/l, respectively. As a reference, NGM medium without compound and NGM medium with DMSO were used.

The L4 worms were incubated until their F1 progeny reached L4 stage (initially overnight at 20°C, followed by 1 day at 15°C and 4 days at 20°C) in the presence of *E.coli* OP50 CS2 as a food source). Thereafter, the amount of F1 progeny with ventral gonads (expressed as percentage of total F1 worms obtained) was determined visually using a microscope.

The results are shown in the Figure. As can be seen, at concentrations of cholesterol >500 µg/l, none of the F1 worms (i.e. with or without dehydroxy-O-methyl-ezetemibe) showed ventral gonads, making it impossible to determine the influence of this compound on cholesterol uptake/transport.

When the worms were incubated at cholesterol concentrations of 150 µg/l, 50 µg/l and essentially zero (no cholesterol added to the medium), the percentage of ventral gonads increased with the concentration of dehydroxy-O-methyl-ezetemibe in the medium.

These results show that at low concentrations of cholesterol, the method of the invention can be used to determine the influence of compounds on cholesterol uptake/transport.

In addition, the amount of F1 progeny obtained ("brood size") at each
5 concentration of dehydroxy-O-methyl-ezetemibe and cholesterol was determined. It was found that at concentrations of cholesterol $>500 \mu\text{g/l}$, the presence of $50 \mu\text{M}$ dehydroxy-O-methyl-ezetemibe has no discernible influence on brood size compared to the reference, whereas at low cholesterol concentrations, the presence of $50 \mu\text{M}$ dehydroxy-O-methyl-ezetemibe severely reduced brood size.

C L A I M S

1. Method for determining the influence of a compound on the uptake of cholesterol by a nematode worm from the medium, comprising the steps of:
 - 5 a) incubating the nematode worm in a suitable medium, wherein said medium contains: (i) the compound; and (ii) cholesterol in a concentration of less than 500 $\mu\text{g/l}$;
 - b) determining at least one cholesterol-relevant phenotype of the nematode worm.
- 10 2. Method according to claim 1, in which the concentration of cholesterol in the medium is less than 400 $\mu\text{g/l}$, preferably less than 350 $\mu\text{g/l}$, more preferably less than 300 $\mu\text{g/l}$.
- 15 3. Method according to any of the preceding claims, in which the cholesterol-relevant phenotype of the worm is chosen from the amount of ventral gonads, growth delay, brood size and/or uptake of labeled cholesterol.
4. Method according to any of the preceding claims, in which the nematode is of the genus *Caenorhabditis*, in particular *C. elegans*.
- 20 5. Method according to any of the preceding claims, in which the nematodes are wild-type *C. elegans* or N2
6. Method according to any of the preceding claims, in which the nematode worms are incubated during at least two different development stages.
- 25 7. Method according to any of the preceding claims, in which the nematode worms are incubated during at least two different generations.
- 30 8. Method according to any of the preceding claims, in which the nematode worms are incubated for a period of at least 2 hours, preferably at least 6 hours, more preferably at least 12 hours, even more preferably at least 24 hours.

9. Method according to any of the preceding claims, in which the nematode worms are incubated for a period of at least 2 days, preferably at least 3 days, and up to 7 days or more.

5 10. Method according to any of the preceding claims, in which the compound is a small molecule.

11. Method according to any of claims 1-9, in which the compound is a nucleic acid.

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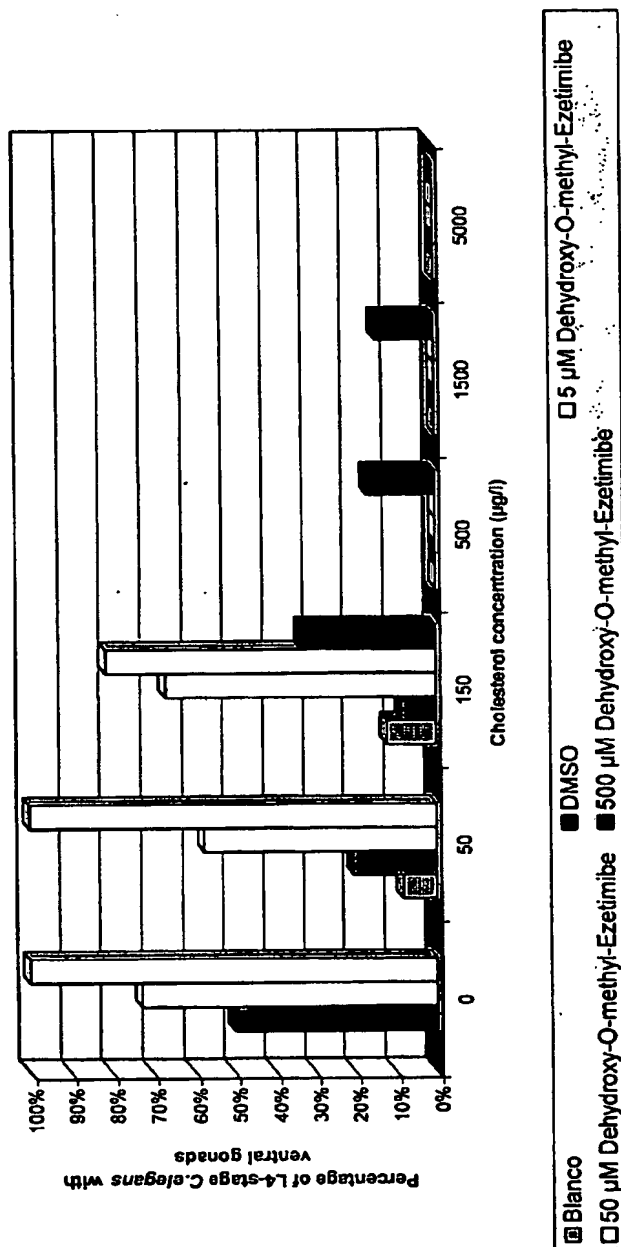
12. Method according to claim 11, in which the nucleic acid is a double stranded RNA molecule.

13. Method according to claim 12, in which said double stranded RNA
15 molecule encodes the nucleotide sequence of (at least part of) a gene from the genome of the nematode worm, such that said double stranded RNA molecule is capable of downregulating said gene in said nematode.

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Figure 1

Effect of Dehydroxy-O-methyl-Ezetimibe and Cholesterol concentration on the presence of ventral gonads in *C. elegans*



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